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The Analysis of Benzodiazepines in Forensic Urine Samples

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16. Abstract The FAA Toxicology and Accident Research Laboratory reports the presence of any drug detected at therapeutic or subtherapeutic levels and the medical condition for which the drug may have been used. Specimens from the pilot of a fatal aviation accident in 1992 were suspected of containing fluoxetine and clonazepam. Initial screening tests using fluorescence polarization immunoassay of a urine specimen revealed 86 ng/ml of a benzodiazepine. Blood from this case was screened using radioimmunoassay and a benzodiazepine was detected at a level in excess of 200 ng/ml. No benzodiazepine was detected in the urine specimen when it was initially tested using HPLC with no hydrolysis of the specimen. Temazepam was eventually identified in urine by HPLC and Mass Spectroscopy, after enzyme hydrolysis of the urine using β -glucuronidase. The blood was found to contain 44 ng/ml of temazepam, 83 ng/ml of fluoxetine, and 138 ng/ml of norfluoxetine. The liver was found to contain 145 ng/ml of temazepam. No tests were performed on the liver for fluoxetine. The identification of temazepam in urine would have been impossible without the enzyme hydrolysis of the specimen prior to extraction and identification. Benzodiazepines are among the most prescribed drugs and a procedure is needed to assure that these drugs will be detected and identified in urine.					
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THE ANALYSIS OF BENZODIAZEPINES IN FORENSIC URINE SAMPLES

INTRODUCTION

Benzodiazepines are commonly prescribed for their anxiolytic, sedative, hypnotic, anticonvulsant, and muscle relaxant properties. Since the introduction of chlordiazepoxide in 1961, more than 3000 compounds have been synthesized and approximately 35 are in clinical use today (1). In 1985, benzodiazepines were the second most identified drug found in urine (2). Only nicotine was found more frequently than benzodiazepines. Benzodiazepines were detected more often than acetaminophen and there were almost twice as many benzodiazepines as cannabinoids found in urine.

Demonstrating the presence or absence of a benzodiazepine in a biological sample can have important legal and medical consequences. Identification of a benzodiazepine in a driver's blood can help corroborate an officer's statement of impairment. The identification of this drug in a person is necessary to ensure compliance with existing laws and prescription orders (3). The use of benzodiazepines by pilots is a violation of FAA regulations. Finding this drug in a pilot may help determine the cause of an accident or compliance with FAA regulations.

Initial screening of a urine specimen collected from the pilot of a fatal aviation accident indicated the presence of a benzodiazepine using fluorescence polarization immunoassay (FPIA). However, no benzodiazepines were detected in urine, when the urine was extracted, without prior hydrolysis, and analyzed using high performance liquid chromatography (HPLC) (Fig. 1). The authors were convinced the specimen did contain a benzodiazepine based on the initial screening results using FPIA, and the fact that temazepam had been identified by HPLC and confirmed by Mass Spectroscopy (MS) in blood. It was postulated that the drug was not being detected because it was present in the form of a glucuronide conjugate. Our approach to the detection of benzodiazepines in urine is presented.

The Structure Activity

The structure of the classical benzodiazepine (Fig. 2) consists of a benzene ring fused to a 7 member diazepine ring. Electron withdrawing groups, such as Cl and NO₂, increase the biological activity. Substitution of a Cl or F in the ortho position of the 5 phenyl ring usually increases the potency and decreases the half-life (e.g., diazepam, temazepam, and flurazepam). Some of the newer benzodiazepines have involved annealing of the 1,2 diazepine position with a triazolo or imidazo ring (e.g., triazolam and alprazolam) (4).

Benzodiazepines are extensively metabolized in the liver. Many of the metabolites are physiologically active. Metabolism can result in N-Alkyl compound formation. Glucuronide conjugates, which are usually inactive, are typically formed by hydroxylated metabolites (1). Temazepam will biotransform, via N-demethylation, to oxazepam and both temazepam and oxazepam metabolize to their glucuronide conjugate (Fig. 3). Temazepam and oxazepam are recovered in the urine primarily as the glucuronide conjugates (5).

Analytical approaches

Urinary benzodiazepines as a group are difficult to identify and quantitate for several reasons (6):

- They are extensively metabolized to the glucuronide conjugate, making it difficult to extract and identify.
- Some of the newer benzodiazepines are more potent, and are found in the urine at low concentrations.
- They tend to be thermally labile during gas chromatography (GC) analysis, unless they are derivatized or converted to their benzophenone form by acid hydrolysis.

Many assays incorporate acid hydrolysis to cleave off the glucuronide for analysis (7); however, the benzodiazepine structure is altered by conversion to a

Figure 1. HPLC Chromatograph of Non-hydrolyzed Urine Sample
Absorbance units versus Retention Time in minutes

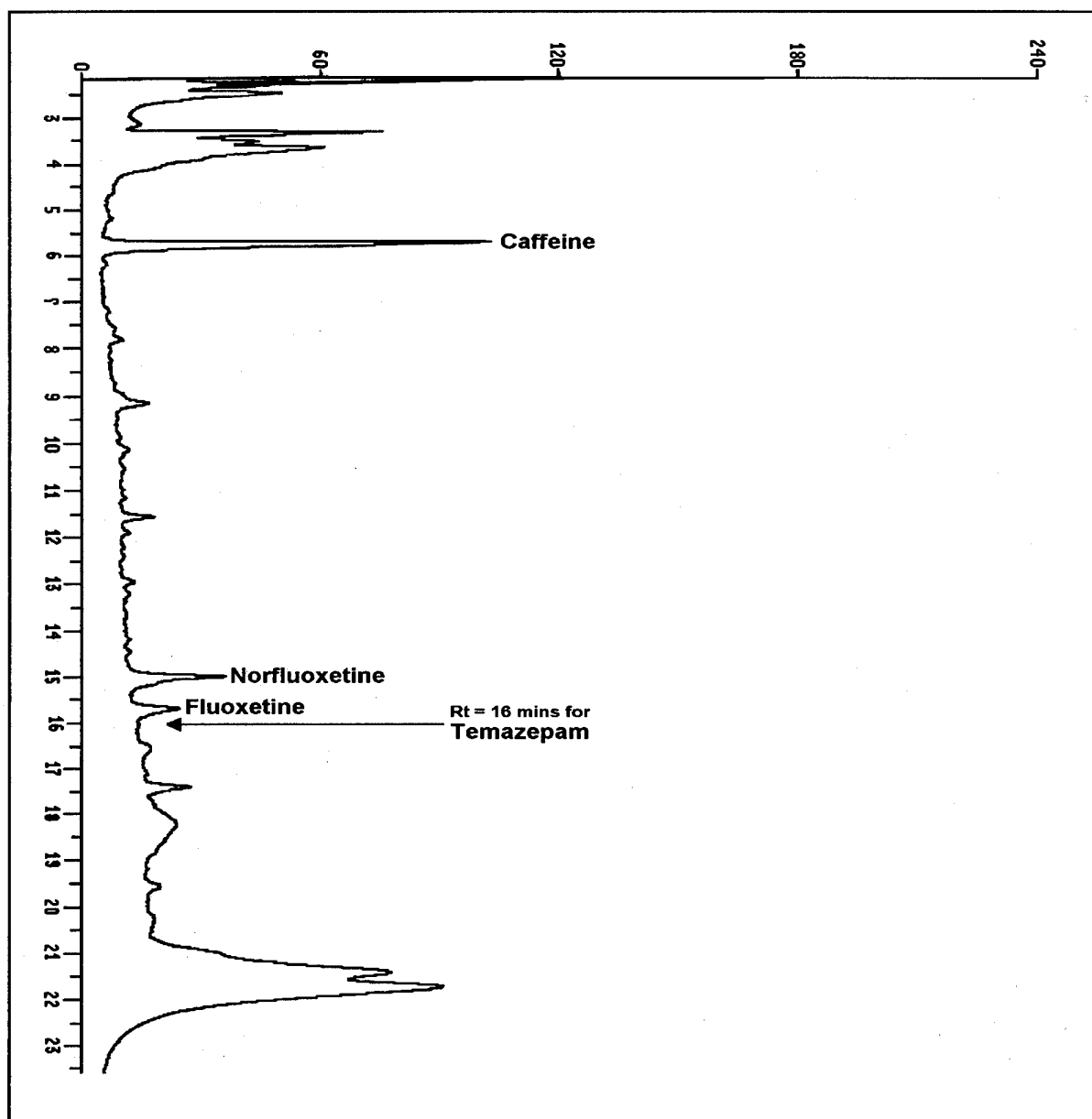


Figure 2. Classical Benzodiazepine structure

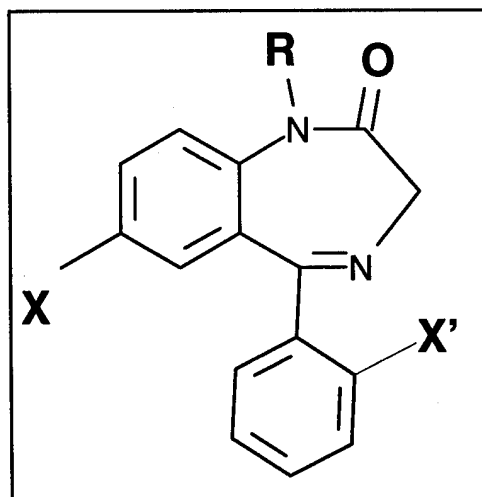


Figure 3. Metabolism of Temazepam

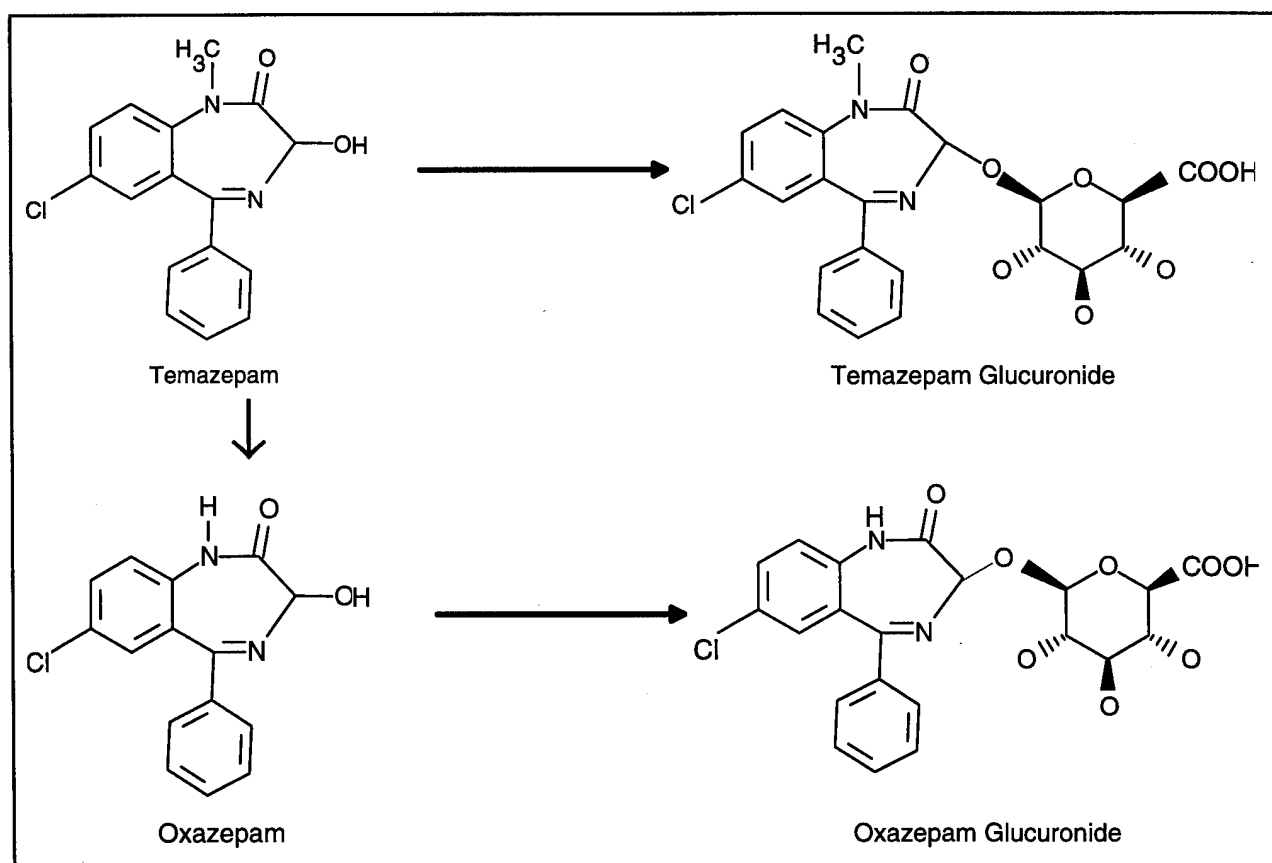


Figure 4. Acid Hydrolysis of Benzodiazepines

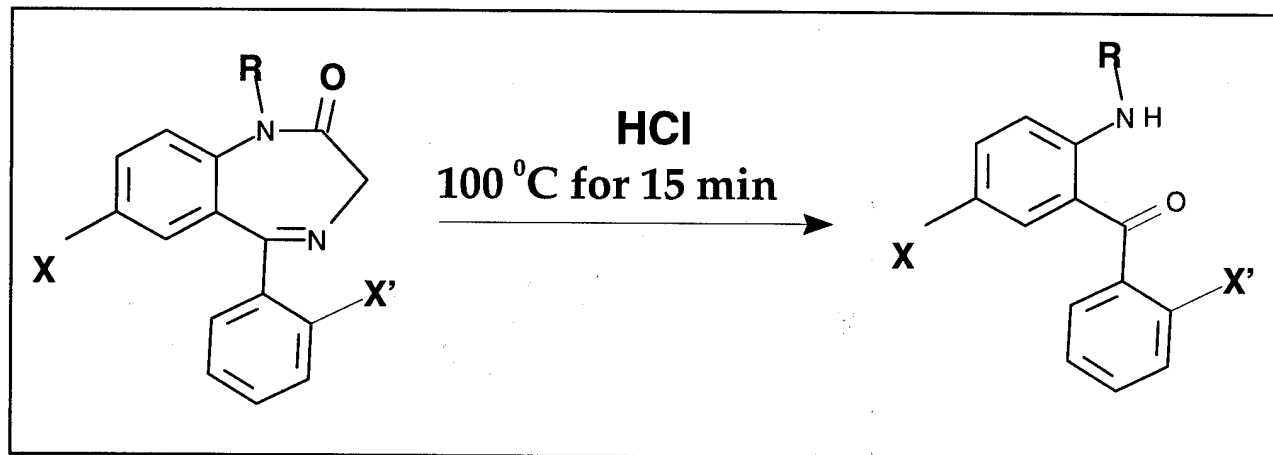


Figure 5. Enzyme Hydrolysis with β - Glucuronidase

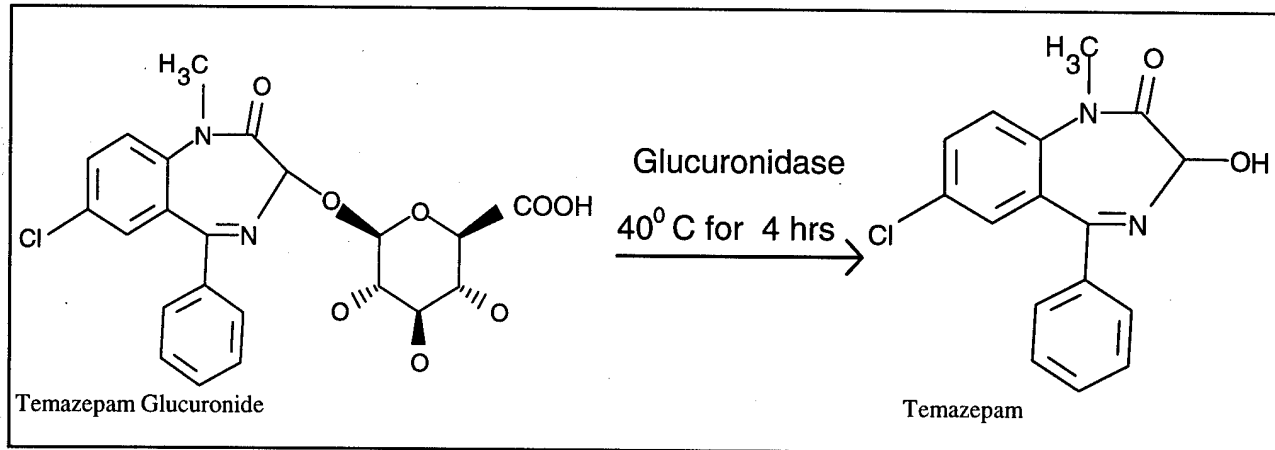


Figure 6. HPLC chromatograph after enzyme hydrolysis of urine specimen.
Absorbance units versus Retention Time in minutes

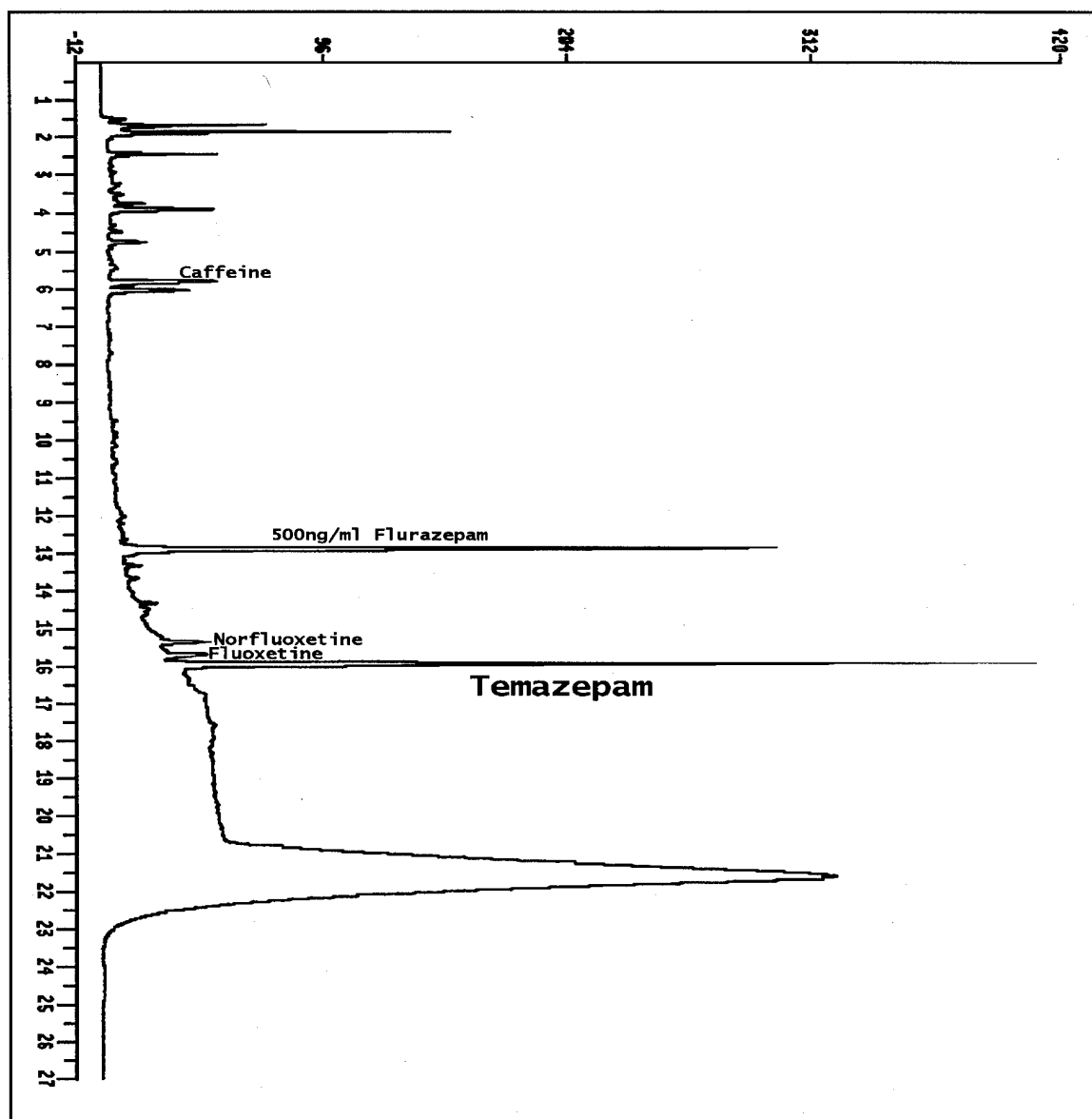
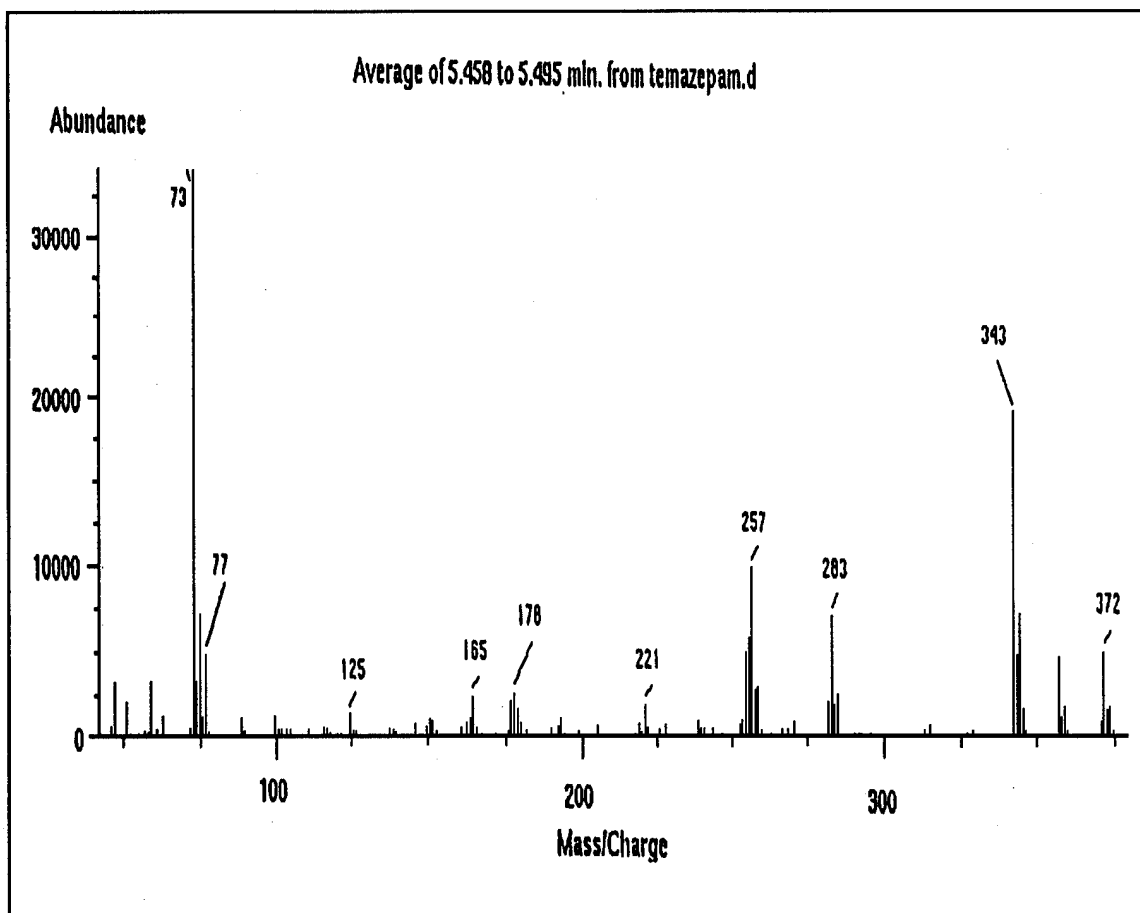


Figure 7. GC/MS Electron Ionization Spectrum of Temazepam



benzophenone during acid hydrolysis, as seen in (Fig. 4). Enzyme hydrolysis, on the other hand, preserves the integrity of the original molecular structure of the benzodiazepine. Enzyme hydrolysis, therefore, makes it possible to identify the starting benzodiazepine (Figs. 5,6) (8). Benzodiazepines accumulate in the urine over time, and for this reason urine is often used initially to screen for benzodiazepines. After finding benzodiazepines in urine one can analyze blood or plasma to identify the specific benzodiazepine previously found in urine.

CAMI Methodology

We have developed a rugged and sensitive method for the analysis of benzodiazepines using enzyme hydrolysis followed by extraction of the analytes using micro disc technology. Samples are confirmed by the use of GC/MS and HPLC. A trimethylsilyl derivative

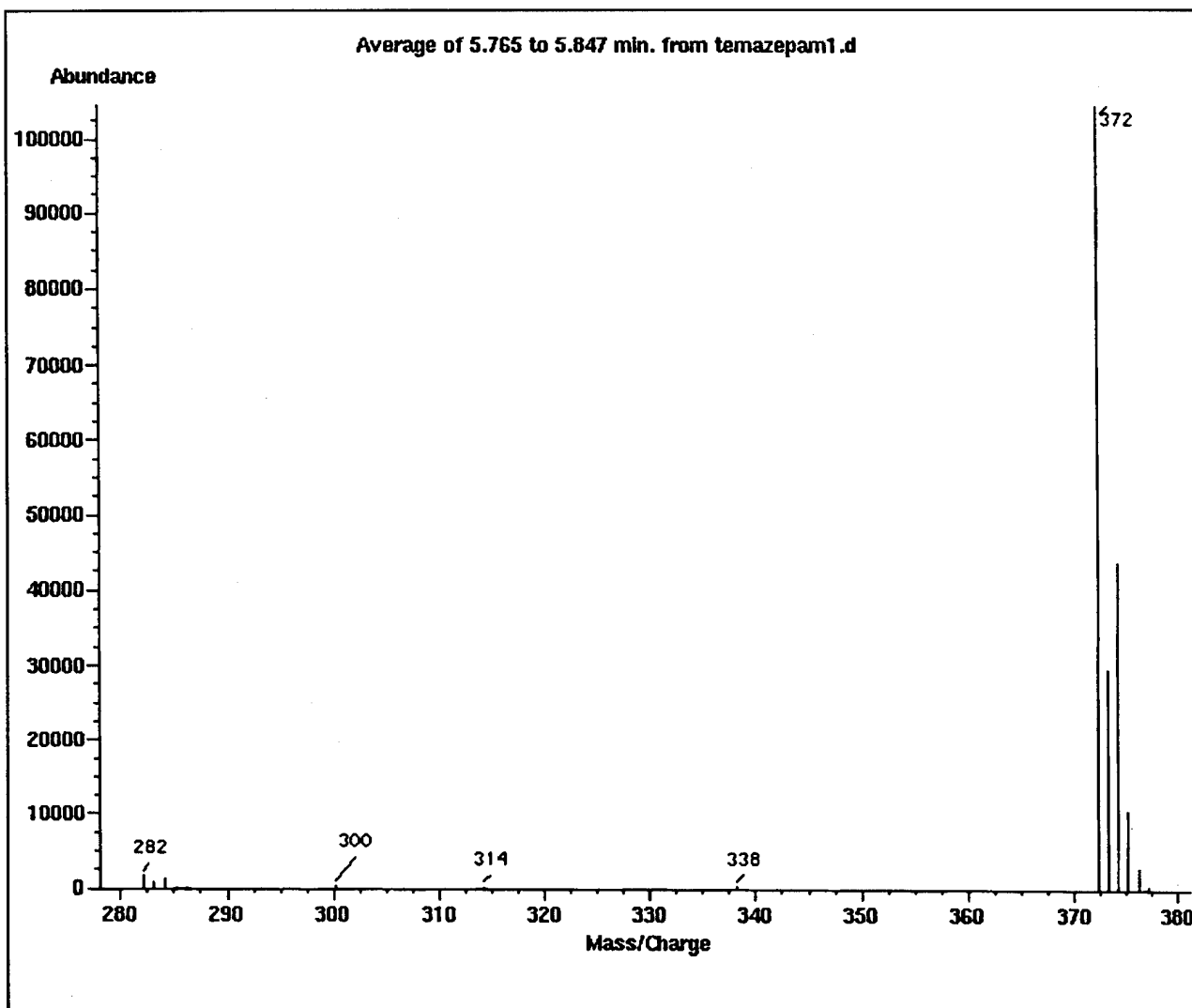
is used to improve the chromatographic characteristics of benzodiazepines for the GC/MS. The mobile phase and gradient program is as described by Logan (9).

MATERIALS AND METHODS

Instrumentation

The HPLC was a Hewlett Packard 1090 Series II, equipped with photodiode array detector and HP auto-injector linked to a Pascal Chemstation. The column was an E. Merck-Lichrospher 60 RP select B, 3 μ m, 250 x 4 mm. The solvents were A.C.S. grade acetonitrile and 10 mM Phosphate buffer, pH 3.2 using a gradient elution with a flow of 1.3 ml/min. The HPLC oven temperature was 35°C. The injection volume 10 μ L monitoring signal 230 nm, from 190 nm to 400 nm.

Figure 8. GC/MS Negative Chemical Ionization Spectrum of Temazepam



The Mass Spectrometer was a Hewlett Packard 5989 MS Engine. The column was a crosslinked 5% phenyl methyl Silicone of 15 meter in length, 0.25 mm internal diameter, and 0.25 μ m film thickness. Helium was the carrier gas with a flow of 1.5 ml/min. The injector, temperature program and transfer line temperatures were maintained at 250 °C, 150-250 °C, and 275 °C, respectively. The acquisition was in electron impact mode scanning from 45 to 480 amu. The injection volume was 1 μ L in the splitless mode.

Sample Treatment

Five mLs of urine is spiked with flurazepam as the internal standard, Two mLs of 0.1M Acetate Buffer pH 5 is added along with 10,000 units b-Glucuronidase to hydrolyze the specimen. The sample was heated for 4 hours at 40 °C. Two mLs boric acid solution and 100 μ L of 11.8 N KOH are then added to pH the sample to 8.5 ± 0.5 .

Extraction

The Micro Disc was prepared by adding 1 mL of methanol to the column reservoir. This is followed by the addition of 1 mL of Borate Buffer, pH 8.5. The sample was poured into the column reservoir and aspirated through the disc. One mL of H₂O is added and aspirated, through as a rinse. The vacuum on the extraction box is increased to dry the disc for 5 minutes. The analyte is eluted off with {CH₂Cl₂ : isopropyl alcohol (80:20) with 2% NH₄OH} into a conical tube.

Derivatization

The eluate was evaporated to dryness, using nitrogen. For analysis on the HPLC, 25 μ L of methanol was added to the tube, vortexed and transferred to an autosampler vial for injection onto the HPLC. For Mass Spectrometer analysis, 50 μ L of BSTFA with 1% TMCS is added to the evaporated eluate tube and incubated for 30 minutes at 70 °C. The tube is allowed to cool and the contents is transferred to an autosampler vial. One μ L is then injected onto the mass spectrometer for confirmation.

Spectra of temazepam were acquired utilizing both electron impact and negative chemical ionization mass spectrometry (NCIMS) (Figs. 7 and 8).

RESULTS

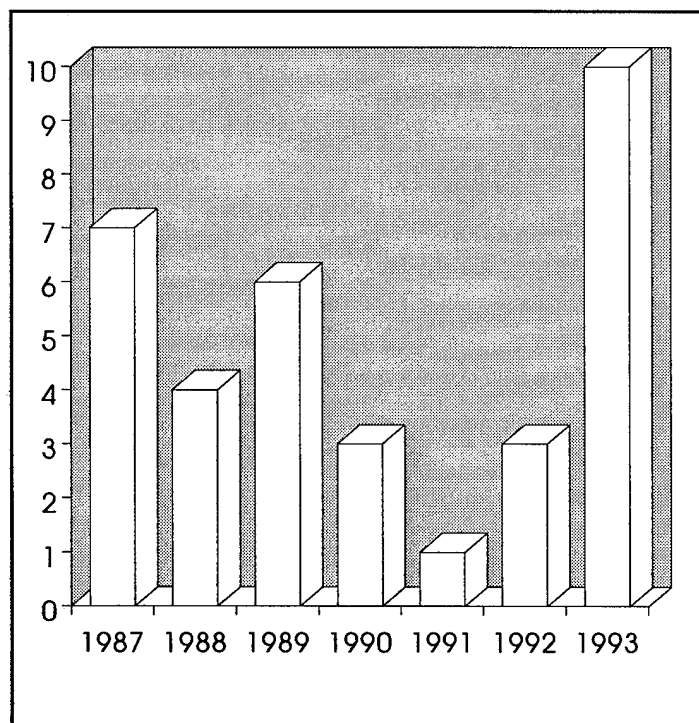
Initial screening tests using fluorescence polarization immunoassay of a urine specimen revealed 86 ng/ml of a benzodiazepine. Blood from this case was screened using radioimmunoassay; a benzodiazepine was detected at a level in excess of 200 ng/ml. The presence of temazepam was detected in the blood using HPLC. No benzodiazepine was detected in the unhydrolyzed urine specimen, when it was initially analyzed, using HPLC at an LOD of 10 ng/ml. Temazepam was identified in urine by the use of HPLC and Mass Spectroscopy, following enzyme hydrolysis of the urine with b-glucuronidase. The blood was found to contain 44 ng/ml of temazepam, 83 ng/ml of fluoxetine, and 138 ng/ml of norfluoxetine. All of these drugs were also confirmed in the urine. Additionally, the liver was found to contain 145 ng/g of temazepam but was not analyzed for fluoxetine or norfluoxetine.

DISCUSSION AND CONCLUSIONS

Both the GC/MS and HPLC were demonstrated to be adequate methods for the determination of a representative benzodiazepine in biological specimens, at subtherapeutic levels. Significant signal to noise ratio above the baseline at the limits of detection (LOD) of 10 ng/mL of temazepam was observed. One can see a large temazepam peak in the post hydrolysis HPLC chromatogram (Fig. 6), when compared to the same urine specimen, which had previously been analyzed without hydrolysis (Fig. 1).

In summary, although the pilot discussed in this report had been prescribed clonazepam, a drug used for the treatment of seizures, the pilot was found to actually be using temazepam, a sleep inducing agent. The prescription medication clonazepam was not found in this case. The finding of fluoxetine and its metabolite, norfluoxetine, confirmed the pilot was taking one of the prescription medications found at the accident. This pilot had not reported to the FAA, as required, any of the drugs found in this case or the medical conditions for which the drugs were being

**Figure 9. Positive Benzodiazepines Cases
[1987-1993]**



taken. This person was flying in violation of FAA regulations, and our toxicological assessment aided in the determination of accident causation.

Since the implementation of the new procedure described in this paper, we have noted an increase in the number of aviation fatality cases reported positive for benzodiazepines (Fig. 9).

The new procedure includes:

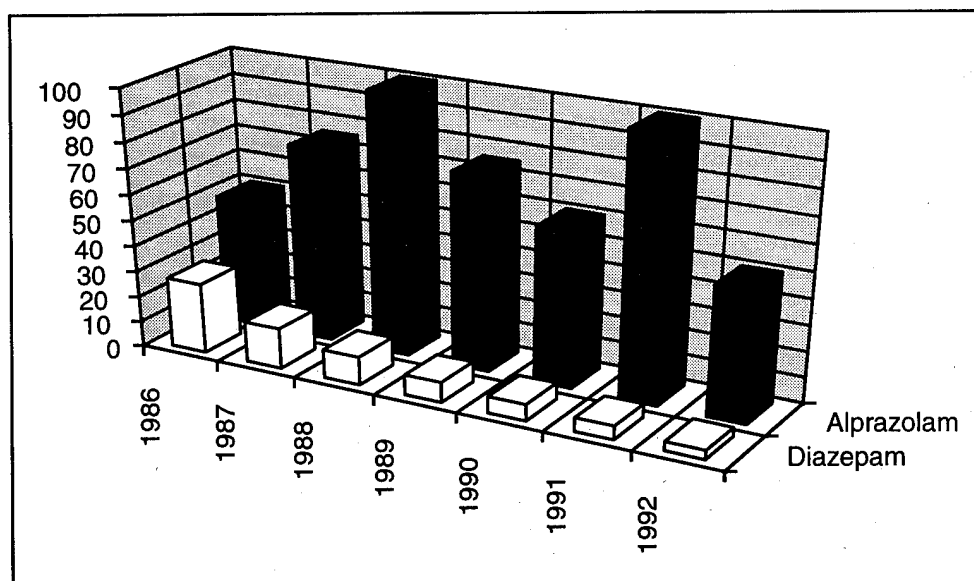
- Urine enzyme hydrolysis
- New extraction methods
- Utilization of HPLC with photo diode array detection
- Confirmation with negative chemical ionization mass spectrometry (NCIMS)

Many of the positive benzodiazepine cases reported in 1993 were found to contain alprazolam and temazepam. The relatively high number of positive

benzodiazepines found in 1987, 1988, and 1989 were most likely related to higher diazepam prescription habits in these years, as noted in Fig. 10.

Diazepam is prescribed in relatively high doses as compared to the newer potent benzodiazepines, such as alprazolam and temazepam. This makes identification of the new benzodiazepines more difficult than the identification of diazepam. Prior to 1992, no cases of benzodiazepines were identified, other than diazepam or its metabolites. There is a direct correlation noted between the decreasing number of diazepam cases being reported, and the decreasing number of prescriptions for diazepam (figures 9 and 10). Although alprazolam has been heavily prescribed, alprazolam was not being frequently detected until 1992 and 1993, when new CAMI analytical methods were implemented.

Figure 10. A Comparison of Diazepam and Alprazolam Use Since 1986



The Y axis represents the percentage of a particular benzodiazepine prescribed during the respective years based on the ranking of the top 200 prescriptions during that year.

Note: The 100 = most prescribed benzodiazepine.

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